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# Cholangiocytes express the aquaporin CHIP and transport water via a channel-mediated mechanism

STUART K. ROBERTS*, MOTOYOSHI YANO*, YOSHIYUKI UENO*, LINH PHAM*, GIANFRANCO ALPINI*, PETER AGRE†, AND NICHOLAS F. LARUSSO*‡

Departments of *Internal Medicine and ‡Biochemistry and Molecular Biology, Mayo Clinic, Rochester, MN 55905; and †Departments of Biological Chemistry and Medicine, Johns Hopkins University, Baltimore, MD 21205

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**ABSTRACT** **Cholangiocytes line the intrahepatic bile ducts and regulate salt and water secretion during bile formation, but the mechanism(s) regulating ductal water movement remains obscure. A water-selective channel, the aquaporin CHIP, was recently described in several epithelia, so we tested the hypothesis that osmotic water movement by cholangiocytes is mediated by CHIP. Isolated rodent cholangiocytes showed a rapid increase in volume in the presence of hypotonic extracellular buffers; the ratio of osmotic to diffusional permeability coefficients was >10. The osmotically induced increase in cholangiocyte volume was inversely proportional to buffer osmolality, independent of temperature, and reversibly blocked by $HgCl_2$. Also, the luminal area of isolated, enclosed bile duct units increased after exposure to hypotonic buffer and was reversibly inhibited by $HgCl_2$. RNase protection assays, anti-CHIP immunoblots, and immunocytochemistry confirmed that CHIP transcript and protein were present in isolated cholangiocytes but not in hepatocytes. These results demonstrate that (*i*) isolated cholangiocytes and intact, polarized bile duct units manifest rapid, mercury-sensitive increases in cell size and luminal area, respectively, in response to osmotic gradients and (*ii*) isolated cholangiocytes express aquaporin CHIP at both the mRNA and the protein level. The data implicate aquaporin water channels in the transcellular movement of water across cholangiocytes lining intrahepatic bile ducts and provide a plausible molecular explanation for ductal water secretion.**

Bile formation by the liver involves secretion of bile by hepatocytes and delivery to a network of interconnecting ducts where bile is modified by cholangiocytes, the epithelial cells that line these conduits inside the liver. Bile secretion by cholangiocytes contributes to total bile flow through the spontaneous and agonist-induced secretion of both ions and water (1). While data have been accumulating on the cellular mechanisms regulating ion transport by cholangiocytes (2–4), the mechanisms regulating water movement across biliary epithelia remain undefined (5, 6).

Conceptually, water may move across biliary epithelia by two pathways: a paracellular pathway between cholangiocytes or a transcellular pathway across both the apical and basolateral cholangiocyte plasma membranes (5, 7). Further, transcellular water movement may occur by simple diffusion across the lipid bilayer or through discrete membrane proteins that form water channels (8). A family of membrane water channels, referred to as aquaporins, was recently identified (9). The aquaporin CHIP [channel-forming integral membrane protein of 28 kDa] is the first characterized molecular water channel (10). When expressed in *Xenopus laevis* oocytes (11) or reconstituted into proteoliposomes (12), CHIP behaves as an osmotically driven, water-selective pore capable of transporting water across the plasma membrane in a rapid, relatively temperature-independent and mercury-sensitive manner. Moreover, immunohistochemical (13–16) and Northern blot (10, 17, 18) analyses and *in situ* hybridization (19) have demonstrated that CHIP has a wide tissue distribution, suggesting that it might be a general water channel (9). Thus, we began to examine the mechanism(s) by which water traverses biliary epithelia.

## MATERIALS AND METHODS

**Cholangiocytes.** Cholangiocytes [>95% pure by specific markers (20)] were isolated from livers of male Fiseher rats (21). For flow cytometry, cholangiocytes were serially incubated with (*i*) 20% normal goat serum, (*ii*) a 1:3 dilution of a mouse monoclonal antibody specific for cholangiocytes (21), and (*iii*) a 1:100 dilution of polyclonal goat anti-mouse IgM conjugated to fluorescein isothiocyanate (FITC; Southern Biotechnology Associates) and identified by their increased FITC fluorescence compared with negative control cells, prepared without incubation with the monoclonal antibody. Over 91% of cells with increased FITC fluorescence were positive for cholangiocyte-specific markers (20). Cell viability was determined by trypan blue exclusion.

**Hepatocytes.** Hepatocytes (>97% pure by morphological appearance) were isolated as described (22).

**Bile Duct Units.** Enclosed polarized bile duct units (BDUs) were prepared from livers of male rats (4).

**Quantitative Phase-Contrast Microscopy.** The size of cholangiocytes in extracellular buffers was measured with an inverted phase-contrast microscope. Freshly isolated cholangiocytes were mounted on a microscope stage in isotonic (300 mOsm) Hepes-buffered saline (HBS: 140 mM NaCl/5.4 mM KCl/0.8 mM $Na_2HPO_4$/25 mM Na Hepes/0.8 mM $MgSO_4$, pH 7.4, 22°C). Cells were exposed to extracellular buffers of differing osmolality (range, 30–300 mOsm); buffers were prepared by diluting HBS with the appropriate volume of distilled water. Serial photographs were taken and cell diameters were measured in a randomized, blinded manner from projected images by using the 4.5-$\mu$m immunomagnetic beads as internal standards. Cell volumes were then derived based on the spherical shape of freshly isolated cholangiocytes (21); results are expressed as percent change in cell volume over time. The osmotic water permeability coefficient ($P_f$, cm/sec) was calculated from osmotic swelling data, initial cholangiocyte volume ($V_0 = 1.56 \times 10^{-9}$ cm$^3$), and surface area ($S = 6.52 \times 10^{-6}$ cm$^2$) (23).

Similarly, enclosed polarized BDUs were exposed to bathing buffers of differing osmolality and the time-dependent change in the *luminal* area was measured from serial photographs by the point-counting method (24).

**Flow Cytometry.** The size of cholangiocytes was also assessed by flow cytometry on a dual-laser flow cytometer

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Abbreviations: FITC, fluorescein isothiocyanate; BDU, intrahepatic bile duct unit; RT, reverse transcription.

(25). FITC fluorescence and 10° incident light scatter of cells were measured (26) and data were analyzed by LYSYS II software (Becton Dickinson).

FITC-labeled cholangiocytes were suspended in isotonic HBS and mounted on the flow cytometer. After baseline values were obtained, cells were exposed to extracellular buffers of 30–300 mOsm and light scatter was measured over the next 30 sec; results are expressed as percent change in light scatter over time.

**Diffusional Water Permeability Studies.** The diffusion permeability coefficient of rodent cholangiocytes was determined experimentally by the linear diffusion technique (27).

**Aquaporin CHIP Gene Expression.** Total cellular RNA was isolated from whole organs and pure preparations of cholangiocytes and hepatocytes (28).

**Reverse Transcription–Polymerase Chain Reaction (RT–PCR).** Specific oligonucleotide DNA primers were based on the rat CHIP DNA sequence (17). With these primers and total cellular RNA as template, cDNA was generated by RT–PCR and sequenced (29).

**RNase Protection Assay.** A 279-bp cDNA corresponding to nt 215–494 of the cDNA encoding rat CHIP was prepared by RT–PCR using total RNA from rat cholangiocytes as template. This cDNA was cloned into the pCR II vector (Invitrogen), and an antisense RNA was transcribed from pCR II as described (30). RNase protection assays were performed (30) with the CHIP antisense RNA probe and total RNA from pure preparations of cholangiocytes and hepatocytes.

**Aquaporin CHIP Protein Expression.** Immunoblotting (13) used a polyclonal anti-CHIP antibody (13) and protein extracted from membranes prepared from human erythrocytes and rat liver cells (31, 32). Immunocytochemistry with preparations of isolated liver cells (21) used an affinity-purified anti-CHIP antibody (0.3 μg/ml) (13) and a Vectastain ABC kit (Vector Laboratories). Staining specificity was confirmed in all specimens by incubations with non-immune rabbit serum and without primary antibody.

## RESULTS

Both techniques used to assess cell size showed that cholangiocytes rapidly increased in size on exposure to hypotonic buffer (Fig. 1). By quantitative phase-contrast microscopy, cholangiocytes exposed to hypotonic (30 mOsm) buffer rapidly increased in size, the cell diameter increasing by up to 38% (i.e., a 165% volume increase) 30 sec after exposure (Fig. 1 *A* and *B*). In contrast, cells exposed to 300 mOsm isotonic buffer remained the same size (Fig. 1 *A* and *B*). These results were confirmed for each buffer osmolality tested by flow cytometric analysis (Fig. 1*C*) of >15,000 cholangiocytes (i.e., >300 cholangiocytes for each time point); maximal cholangiocyte swelling in hypotonic (30 mOsm) buffer was not obtained after 5 min of exposure (data not shown), suggesting that spontaneous volume regulation was not significantly contributing to the volume response over this time period. Extracellular buffer osmolality had a significant inverse effect ($P < 0.0001$, ANOVA) on the magnitude of the increase in cholangiocyte volume and on the magnitude of the decrease in light scatter (Fig. 1 *B* and *C*). Thus, analysis by phase-contrast microscopy of individual cells and by flow cytometry of large numbers of cells both demonstrated that cholangiocytes are capable of rapid transmembrane water movement in response to osmotic buffers.

From the initial slope of the curves generated in Fig. 1*B*, the calculated osmotic permeability coefficient ($P_f$), of normal rat cholangiocytes was 0.005 cm/sec. By comparison, the diffusional permeability coefficient ($P_d$) of normal rat cholangiocytes calculated from the bulk diffusion coefficients for $^3H_2O$ in packed cholangiocytes (<0.245 $cm^2$/sec), extracellular (supernatant) fluid (2.096 $cm^2$/sec), and intracellular medium (0.715 $cm^2$/sec) and the relative extracellular volume

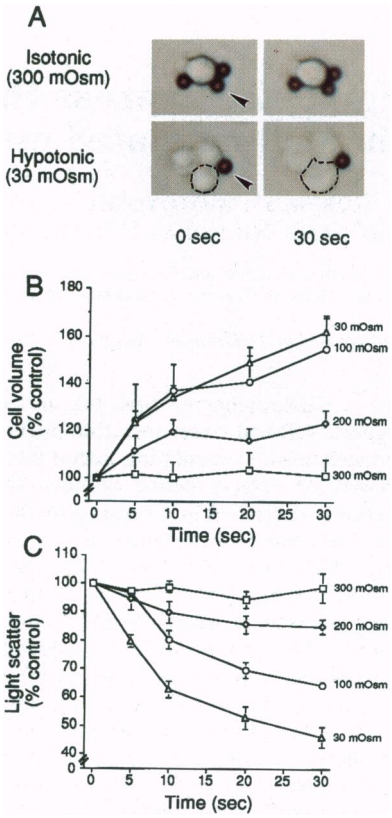


FIG. 1. Osmotic water transport in rodent cholangiocytes. (*A*) Phase-contrast micrographs of purified cholangiocytes in isotonic (*Upper*) and hypotonic (30 mOsm) (*Lower*) buffers. Note the immunomagnetic beads attached to cholangiocytes (arrowheads). Cells in hypotonic buffer are outlined for ease of size comparison. (*B* and *C*) Time course of osmotic swelling of cholangiocytes. Cells were exposed to either 300 mOsm (□), 200 mOsm (◇), 100 mOsm (○), or 30 mOsm (△) buffer. (*B*) Cholangiocyte size assessed by quantitative phase-contrast microscopy. Results reflect measurements of >16 cholangiocytes for each time point. (*C*) Cholangiocyte size assessed by light scatter with a flow cytometer. Results reflect measurements from at least four separate experiments.

(0.133) was $<5 \times 10^{-4}$ cm/sec. Thus, the ratio of osmotic to diffusional water permeability ($P_f/P_d$) is >10. Given that an established criterion for channel-mediated water transport is a $P_f/P_d$ ratio >1 (9, 33), these data suggested that osmotically induced water movement by cholangiocytes was mediated by membrane water channels. To pursue this possibility, we studied the effects of both temperature and mercury on water transport by isolated cholangiocytes. For both variables, results are given for studies with flow cytometry because of the larger number of cells analyzed.

The time-dependent decrease in light scatter by cholangiocytes in a range of hypotonic buffers was not different between experiments done at 22°C and those done at 4°C (Fig. 2). Control data at both temperatures for cells in isotonic buffer are also shown. Thus, temperature had no effect on the transmembrane transport of water by cholangiocytes.

Preincubation of cholangiocytes with $HgCl_2$ (0.3 mM) significantly inhibited the time-dependent decrease in light scatter by cholangiocytes in hypotonic (30 mOsm) buffer. This inhibitory effect of $HgCl_2$ was reversible; addition of 2-mercaptoethanol to $HgCl_2$ during the incubation period blocked the effect of $HgCl_2$ on water movement (Fig. 3*A*). Addition of 2-mercaptoethanol alone did not effect the cholangiocyte volume response (data not shown). Moreover, inhibition of water movement by $HgCl_2$ was dose-dependent (0.1–3 mM); the magnitude of the change in light scatter of cholangiocytes in 30 mOsm buffer increased with decreasing

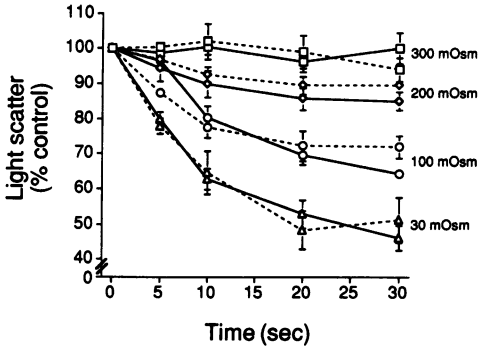


FIG. 2. Effect of temperature on osmotic water transport by cholangiocytes assessed by flow cytometry. Cells were exposed to either 300 mOsm (□), 200 mOsm (◇), 100 mOsm (○), or 30 mOsm (△) extracellular buffer. Experiments were performed at 22°C (——) and 4°C (----). Results reflect measurements from at least four separate experiments.

concentrations of $HgCl_2$ (Fig. 3*B*). Furthermore, inhibition by $HgCl_2$ was evident over a range of hypotonic osmotic gradients (Fig. 3*C*). Exposure of cholangiocytes to a small (15 mM) osmotic gradient also resulted in rapid and significant ($P < 0.05$) cell swelling (Fig. 3*C*). Morphology and viability of cholangiocytes were not significantly affected by $HgCl_2$; cholangiocyte viability was 92% before and 87% after 10 min of incubation with 3 mM $HgCl_2$.

Studies in isolated enclosed BDUs provided additional evidence for a channel-mediated mechanism of water movement by cholangiocytes (Fig. 3*D*). By quantitative phase-contrast microscopy, the luminal area of BDUs rapidly and significantly ($P < 0.05$) expanded by 20.1 ± 5.0% in the first 30 sec after BDUs were exposed to hypotonic (150 mOsm) bathing buffer; in contrast, BDUs in isotonic buffer showed no change. BDUs exposed to 30 mOsm buffer actually ruptured within the first 30 sec. Moreover, preincubation of BDUs with $HgCl_2$ (0.3–3 mM) blocked the increase in luminal area due to hypotonic buffer. As expected, this effect of $HgCl_2$ was reversible; addition of 2-mercaptoethanol blocked the inhibitory effect of $HgCl_2$ on water movement (Fig. 3*D*). In addition, preincubation with protamine, (300 μg/ml), which blocks the paracellular pathway in certain tissues (34), did not significantly effect water movement across BDUs (data not shown). These data show that water moves rapidly across polarized cholangiocytes into the lumen of enclosed BDUs in response to osmotic buffers by a $HgCl_2$-inhibitable, protamine-independent process, thus reflecting a transcellular rather than a paracellular pathway. Since the data in Figs. 1–3 strongly suggested that the principal mechanism regulating transcellular water movement by cholangiocytes was via water channels in the plasma membrane, we explored which water channel(s) might be responsible for this functional activity.

Gel electrophoresis of products obtained by RT–PCR using specific DNA primers for rat CHIP showed a band at 300 bp in the lane where RNA isolated from purified cholangiocytes was used as template (Fig. 4*A*). This band was identical in location to that obtained with whole kidney RNA as template, our positive control. No band was detected when an equal amount of RNA from purified hepatocytes was used as template. Further, by DNA sequencing, the band obtained was 100% homologous to the rat CHIP cDNA sequence.

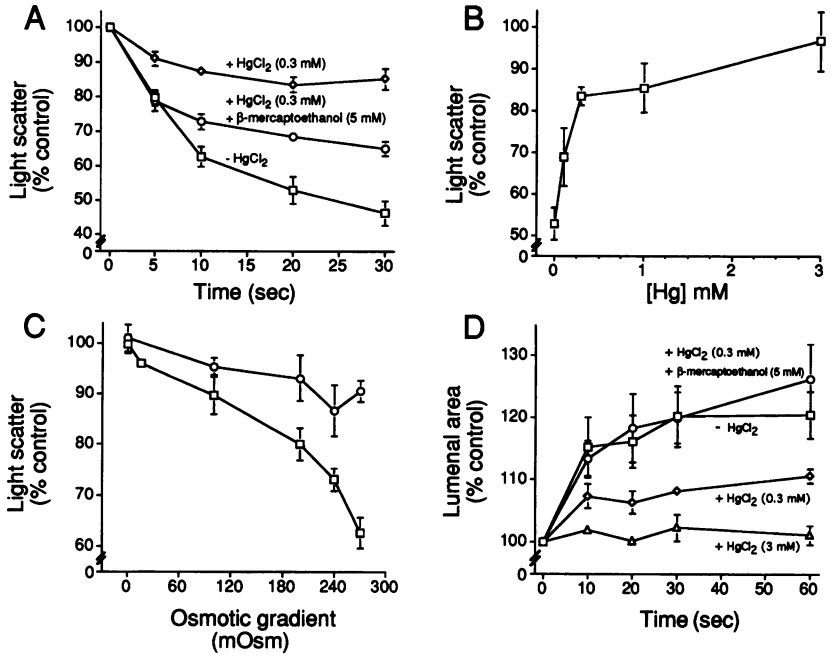


FIG. 3. Effect of $HgCl_2$ on osmotic water transport by cholangiocytes. (*A*) Effect of $HgCl_2$ on the time course of osmotic swelling of cholangiocytes assessed by flow cytometry. Cells were exposed to 30 mOsm buffer in the absence of $HgCl_2$ (□) ($n = 8$) or after a 10-min preincubation with 0.3 mM $HgCl_2$ (◇) ($n = 8$). Cholangiocytes were also studied after preincubation for 5 min with 0.3 mM $HgCl_2$ followed by 10 min with 5 mM 2-mercaptoethanol and 0.3 mM $HgCl_2$ (○) ($n = 4$). (*B*) Dose–response effect of $HgCl_2$ on the osmotic swelling of cholangiocytes assessed by flow cytometry. Cells were preincubated for 10 min with various concentrations of $HgCl_2$ and then exposed to 30 mOsm buffer. Results represent light scatter values 20 sec after exposure to hypotonic buffer and reflect measurements from at least four separate experiments. (*C*) Relationship between extracellular osmotic gradient and $HgCl_2$ inhibition of cholangiocyte swelling assessed by flow cytometry. Light scatter was measured in cells after exposure to various extracellular osmotic gradients in either the absence (□) or the presence of 3 mM $HgCl_2$ (○). Results represent light scatter values 10 sec after buffer exposure and reflect measurements from at least three separate experiments. (*D*) Effect of $HgCl_2$ on the time course of osmotic swelling of the lumen of enclosed BDUs assessed by quantitative phase-contrast microscopy. BDUs were exposed to hypotonic (150 mOsm) buffer either in the absence of $HgCl_2$ (□) ($n = 5$) or after a 10-min preincubation with 0.3 mM $HgCl_2$ (◇) ($n = 6$) or 3 mM $HgCl_2$ (△) ($n = 6$). Studies were also done after BDU preincubation for 5 min with 0.3 mM $HgCl_2$ followed by 10 min with 5 mM 2-mercaptoethanol and 0.3 mM $HgCl_2$ (○) ($n = 3$).

Thus, the data strongly suggest that normal rat cholangiocytes, but not hepatocytes, contain the transcript for CHIP.

To confirm this, transcript levels were directly assessed by RNase protection assay (Fig. 4*B*). Due care was taken to utilize equal amounts of total RNA for each liver cell type. As expected, the transcript was present in whole kidney, our positive control, and absent in whole brain, our negative control. Further, as predicted, the transcript was present in normal cholangiocytes but not in hepatocytes. These data confirm the results obtained by RT–PCR and demonstrate that normal rat cholangiocytes but not hepatocytes express the transcript for CHIP.

When membranes prepared from red blood cells and liver cells were analyzed by immunoblotting with a rabbit polyclonal antibody directed against the C-terminal, cytoplasmic domain of human CHIP (13) (Fig. 4*C*), a band at 28 kDa was detected in the lane containing protein from membranes prepared from isolated rat cholangiocytes. This band was identical in location to that obtained from human erythrocytes, our positive control. No band was detected when equal amounts of protein extracted from hepatocyte membranes were analyzed.

Confirmation of CHIP protein expression in rat liver epithelial cells was obtained by immunocytochemistry using an affinity-purified anti-CHIP antibody. Reaction product was clearly detected in purified cholangiocytes, with >90% of cholangiocytes staining positive for CHIP (Fig. 5*A*). No reaction product was seen in cholangiocytes stained with nonimmune serum (Fig. 5*B*). Further, no reaction product was detected in normal hepatocytes stained with either the anti-CHIP antibody or nonimmune serum (Fig. 5 *C* and *D*). These data provide conclusive evidence that normal cholangiocytes, but not hepatocytes, express CHIP protein.

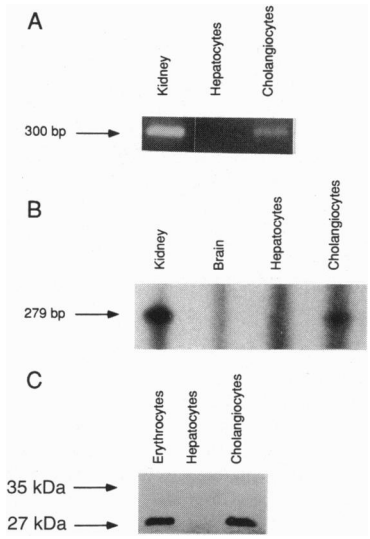


FIG. 4. Aquaporin CHIP expression in rodent cholangiocytes. (*A*) Gel electrophoresis of products obtained by RT–PCR using primers specific to the rat CHIP gene. For each reaction, 1 μg of total RNA was used as template. (*B*) RNase protection assay for CHIP transcript. For each liver cell type, 15 μg of total RNA was loaded while 10 μg of total RNA from kidney (positive control) and whole brain (negative control) was loaded. The signals shown were obtained following 64 hr of exposure. (*C*) Immunoblot for CHIP on membranes prepared from human erythrocytes and rat liver cells. Note that while equal amounts (15 μg) of protein extracted from cholangiocyte and hepatocyte membranes were loaded, the amount of erythrocyte membrane protein loaded was only 0.1 μg. The signals shown were obtained after 4 min of chemiluminescence.

## DISCUSSION

To our knowledge, this is the first study of osmotic water permeability in cholangiocytes. By two independent techniques, quantitative phase-contrast microscopy and flow cytometry, we studied the kinetics of osmotic-induced water movement by cholangiocytes and the mechanism of water movement involved, using the effects of both temperature and mercury to differentiate simple diffusion from channel-mediated transport. We also directly measured the diffusional permeability coefficient of cholangiocytes. Our major findings are that (*i*) cholangiocytes rapidly increase in size in response to hypotonic buffers, the magnitude of the increase being inversely proportional to buffer osmolality; (*ii*) temperature has no effect on the time-dependent increase in size of cholangiocytes in hypotonic buffers; (*iii*) $HgCl_2$ inhibits the osmotically induced increase in cholangiocyte size in a reversible and dose-dependent manner; and (*iv*) the value of the osmotic/diffusional water permeability ratio for cholangiocytes is >10. These observations indicate that isolated cholangiocytes are capable of rapid transmembrane water movement in response to osmotic gradients via a mechanism consistent with transport through discrete membrane proteins that form water channels. Still, while isolated cholangiocytes were suitable for assessing both the kinetics of and the principle mechanism involved in transmembrane water movement, studies with enclosed BDUs were necessary to begin to address the more physiologically relevant questions of whether and by what mechanism water actually moves across an intact layer of polarized cholangiocytes. Indeed, our observation that the luminal area of enclosed BDUs increases after exposure to hypotonic buffer by a process which is protamine-independent and reversibly inhibited by $HgCl_2$ has two physiologically important implications: (*i*) a transcellular rather than a paracellular pathway plays an important role in osmotically induced transepithelial water movement by biliary epithelia *in vivo* and (*ii*) the mechanism regulating this transcellular water movement *in vivo* is via membrane water channels. Further, the demonstration of rapid transmembrane water movement by cholangiocytes after exposure to a small (15 mOsm) and likely more physiological gradient strengthens the possibility that channel-mediated water movement is important *in vivo*.

Having generated data consistent with the presence of a water channel in rodent cholangiocytes, we next addressed

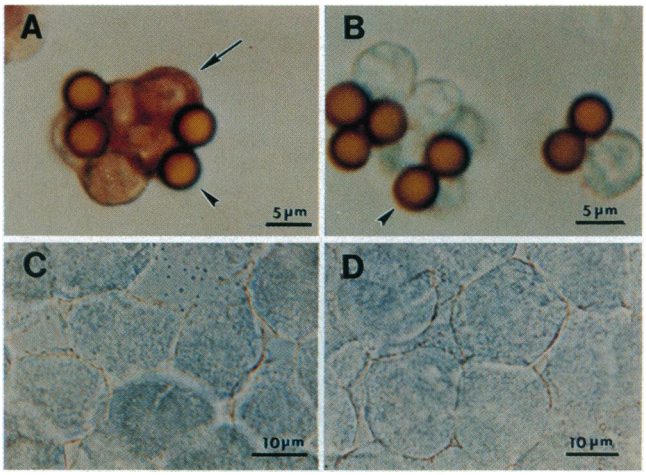


FIG. 5. Immunocytochemical assessment of CHIP protein expression in rat liver epithelial cells. (*A* and *B*) Light micrographs of purified rat cholangiocytes stained with an affinity-purified polyclonal IgG antibody to CHIP (*A*) or with nonimmune serum (*B*). Note the immunomagnetic beads attached to cholangiocytes (arrowheads) and membranous staining of CHIP in cholangiocytes stained with the anti-CHIP antibody (arrow). (*C* and *D*) Light micrographs of purified rat hepatocytes stained with affinity-purified anti-CHIP antibody (*C*) or with nonimmune serum (*D*).

the question of which water channel might be responsible. We speculated that aquaporin CHIP was involved, based on immunohistochemical studies of human liver by Nielsen *et al.* (15). Using combined molecular and immunological approaches, we demonstrated that normal rat cholangiocytes express both the transcript for aquaporin CHIP and the protein itself. These results agree with the findings that CHIP protein is expressed in cholangiocytes from other mammals including humans (15) and guinea pigs (S.K.R., P.A., N.F.L., unpublished data). Thus, the data show that rat cholangiocytes contain at least one candidate water channel potentially responsible for transmembrane water movement. Although we have not unequivocally demonstrated that CHIP is the protein responsible for transcellular water transport by cholangiocytes, the evidence is suggestive. The high osmotic/diffusional water permeability coefficient ratio, temperature independence, and reversible mercury sensitivity of transcellular water movement by cholangiocytes are all consistent with data from studies of CHIP in *Xenopus* oocyte (11) and liposome (12) expression systems.

The presence of CHIP at the apical and, in some cases, the basolateral domain of several epithelia intimately involved in fluid secretion supports a physiologic role for CHIP in transmembrane water flow throughout the body (13–16). Similarly, the presence of CHIP at the apical and basolateral domains of cholangiocytes, together with the demonstration of a functional water channel in rodent cholangiocytes, suggests that CHIP plays an important role in ductal bile formation. At the apical (luminal) membrane, CHIP may contribute to ductal water secretion by rapidly transporting water into the lumen in response to transient osmotic gradients. Although the actual transmembrane osmotic gradients involved are unknown, these osmotic gradients are most likely created by the hormone-stimulated transport of ions across the luminal membrane. At the basolateral membrane, the role of CHIP is more speculative. The demonstration of CHIP in the basolateral domains of both cholangiocytes and endothelial cells of the peribiliary capillaries of both humans (15) and rodents (data not shown) suggests a functional relationship between the two cell types. This relationship may involve the rapid transport of plasma water from the peribiliary capillaries to the biliary epithelial cells during basal and agonist-stimulated ductal secretion. Rapid movement of plasma water across both the basolateral and apical membranes of cholangiocytes would allow the relative isoosmolar status of the cell to be maintained even under choleretic conditions.

Given that CHIP is likely to be an important mediator of water movement by cholangiocytes during ductal bile secretion, the relationship between hormones known to regulate ductal water secretion (e.g., secretin and somatostatin) (30, 35) and the functional activity of CHIP is of interest. In contrast to the vasopressin-responsive water channel of renal epithelial cells, which recycles between the plasma membrane and an intracellular compartment of vesicles (36), CHIP is thought to permanently reside in the plasma membrane and to be constitutively active in response to osmotic gradients (9). While the proposed physiologic role(s) of CHIP in cholangiocytes outlined above is consistent with this model, additional studies are necessary to directly address both the subcellular location and the physiologic regulation of CHIP in cholangiocytes as they relate to hormone-induced ductal bile secretion.

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